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PRINCIPAL INVESTIGATOR: Ian de Belle, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

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Ian de Belle, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Burnham Institute
La Jolla, California 92037

E-Mail: idebelle@burnham.org

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This project is designed toward the identification of a comprehensive set of target genes for the transcription factor Egr1. Since breast cancer cells often do not express Egr1 while normal breast epithelial cells do, it is important to define Egr1 target genes with the hope that critical patterns of Egr1 regulated gene expression active only in normal cells will be revealed. I have chosen to approach this project using an in vivo crosslinking and chromatin immunoprecipitation (ChIP), protocol.

Using this approach I have successfully cloned a newly identified Egr1 target gene called TOE1. This gene is currently being characterized, but has the property of an inhibitor of cellular growth when overexpressed. Furthermore, TOE1 may act through interaction and modification of the activity of p53.

I have furthered the search for Egr1 target genes using the ChIP approach by adapting an array hybridization protocol using custom generated mammalian gene promoter arrays using a high throughput approach.

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Introduction

This annual report provides a detailed description of my research activities and accomplishments for the past year of funding support from the USAMRMC Breast Cancer Research Program. The project has as its major goal to define important target genes for the Egrl transcription factor in breast cells using the ChIP approach. With Egrl being a primary response gene that is expressed in normal, but not rarely in breast cancer cells, identification of its gene targets represents an important means by which potentially important transcriptional deficiencies in breast cancer cells may be discovered.

Here, I present an update on my further characterization of my newly cloned Egrl target gene TOE1. Also, I present my progress in expanding the high throuput analysis of Egrl target genes using custom made mammalian gene promoter arrays. I have attached a published manuscript providing detailed experimental methods for the cloning and partial characterization of TOE1.

Body

The results presented herein are divided into two sections. The first details the progress on TOE1 characterization. My initial cloning and partial characterization of TOE1 is presented as a published manuscript attached to provide background details. The second section presents my approach and progress in the development of an array based high throughput screen for Egrl targets.

Part 1: Further characterization of TOE1 (Target Of Egrl)

In my previous report I presented experimental evidence that TOE1 is localized within the nucleus and nucleolus of cells. I also showed that TOE1 is incapable of directly activating transcription suggesting that it is not an independently acting transcription factor. Further, I presented evidence that TOE1 is capable of interacting with the tumor suppressor protein p53 by co-immunoprecipitation. Here, I present results suggesting that the interaction between TOE1 and p53 is enhanced following cellular exposure to uv irradiation, a known activator of p53 activity.

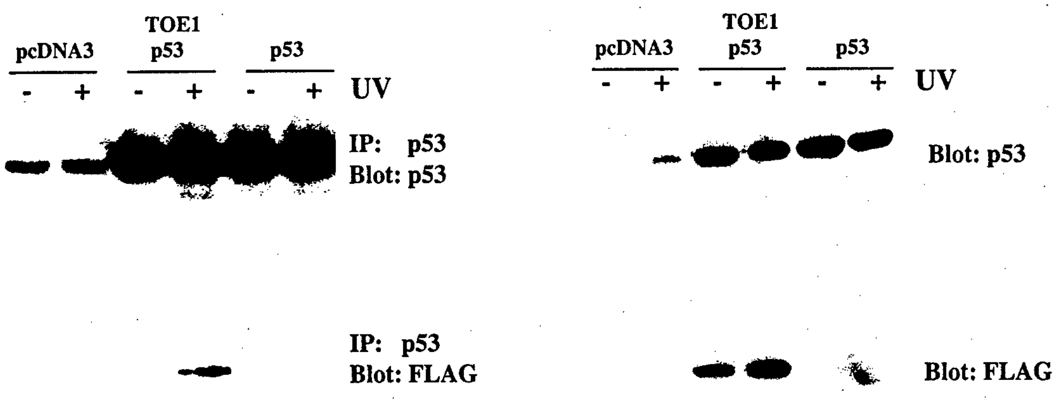

Figure 1. Co-immunoprecipitation of TOE1 with p53 following uv irradiation
293 cells were transfected with empty vector (pcDNA3), or vectors expressing p53 alone or together with FLAG-tagged TOE1. Following transfection cells were either untreated or exposed to 40 J/m^2 uv and incubated for 2h at 37 °C. Cell lysates were immunoprecipitated with anti-p53 antibodies, and western blots probed for either p53 or FLAG-TOE1. The left panels show the immunoprecipitates and the right panels show the presence of the transfected and expressed proteins from whole cell lysates.

Figure 1 shows an enhanced interaction between TOE1 and p53 following uv irradiation. To investigate whether this interaction has a functional consequence with respect to the activity of p53, I examined the transactivating potential of p53 in the presence or absence of TOE1. Since I had previously found that cells expressing TOE1 were growth inhibited and had high levels of p21 expression, and since p21 is a known target for p53, I chose to examine the activation of the p21 promoter in a reporter assay. As seen in figure 2, while expression of p53 was able to activate the reporter, co-expression with TOE1 significantly enhanced this activation. This result, together with the co-immunoprecipitation result suggests that TOE1 may function as a modifier of p53 transcriptional activity through interaction.

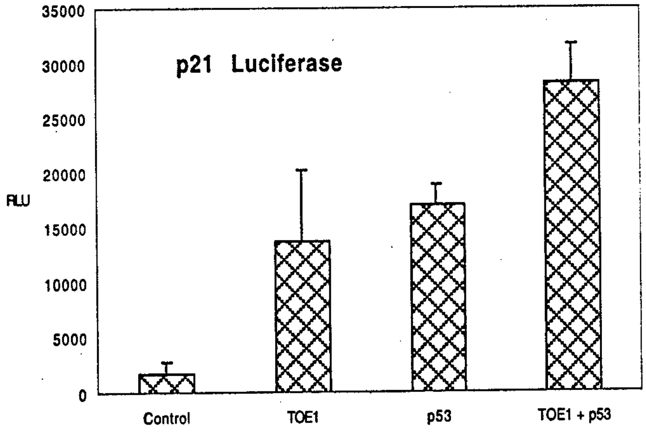

Figure 2. Transactivation of the p21 promoter by p53 and TOE1
293 cells were transfected with a luciferase reporter vector containing the p21 promoter together with the indicated expression vectors. Control samples were transfected with empty expression vector. 24 hours after transfection, cells were collected and assayed for luciferase activity by luminometry. Results are presented as the average of tripicate samples with the standard deviations indicated with error bars.

In order to better understand the possible regulation of the activity of p53 by TOE1, I reasoned that uv irradiation may result in a change in post translational modification of TOE1 to promote interaction. To test this possibility, I incubated TOE1 transfected cells with radiolabeled orthophosphate in cells either untreated or uv irradiated. Following a two hour incubation after irradiation, cells were lused and TOE1 immunoprecipitated with anti-TOE1 antibodies. As shown in figure 3, TOE1 is indeed modified by phosphorylation following uv irradiation. I am currently working on determining whether the phosphorylation of TOE1 is critically important for its interaction with p53, or if phosphorylation may enhance the transcriptional activity of the TOE1/p53 complex..

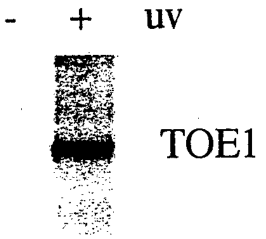

Figure 3. TOE1 is a phosphoprotein.
HT1080 cells were transfected with a TOE1 expression vector, and 24 hours later one dish of cells was exposed to 40J/m^2 uv irradiation while a second dish left untreated. The cells were labeled with ^{32}P inorganic phoshate for 2 hours and then extracted and immunoprecipitated with TOE1 antibodies. Phosphorylated TOE1 is seen only following exposure to uv irradiation.

In order to accomplish this it is necessary to identify the phosphorylation site(s) on TOE1 so that they can be mutated and then tested for functional activity. The first step in this process is to map the site(s). To this end I have performed tryptic peptide mapping of phosphorylated TOE1. As shown in figure 4, phosphorylated TOE1 displays a discreet set of three phosphopeptides migrating in close proximity to each other.

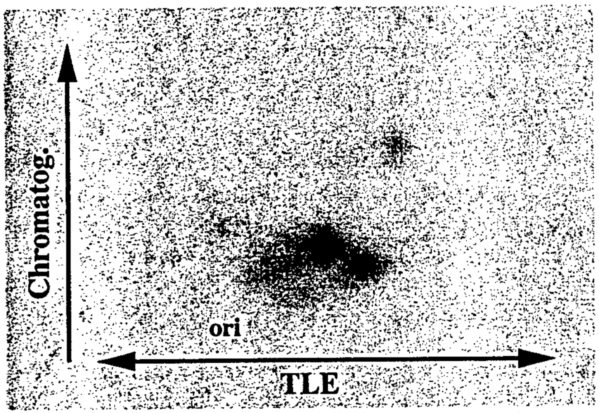

Figure 4. TOE tryptic peptide map.
Phosphorylated TOE1 was subjected to digestion by trypsin and then mapped by horizontal thin layer electrophoresis at pH 1.9. The map was then subjected to ascending chromatography in a pyridine based system and the chromatogram autoradiographed for 2 days to visualize the phosphopeptides. Ori. : origin of TOE1 spotting.

Fine mapping of TOE1 phosphorylation sites will be accomplished by extracting the phosphopeptides from the chromatogram and subjecting the eluted peptides to MALDI-TOF mass spectroscopy. These studies are presently ongoing, and peptides are being accumulated to provide sufficient material for an accurate spectrum to be determined. Once the sequence of the peptides has been determined, likley phospho –acceptor sites will be mutated by standard site -directed mutagenesis, and then transfected into mammalian cells to probe for functional deficiencies.

Part 2: Expansion of the mammalian promoter array

To allow an array based method of identification of Egr1 target genes, I have proceeded to generate a promoter array. In my previous report, I presented the idea and preliminary production of this type of array. At that time I had successfully amplified a set of approximately 85 mammalian gene promoters. In order to expand this set, I set out to amplify a much larger set of promoters using a 96 well PCR approach. I felt that this would be necessary to generate an array containing a significant coverage of human promoter genome. To this end, I have tooled up the amplification in a 96well format and have achieved a 80-90% success rate for amplification. An example of a typical amplification test is shown in figure 5. As it can be seen, this high throughput mode of human promoter amplification yields good PCR products, which are then cleaned through a multiplate filter, and then placed in 50% DMSO for array spotting. To date, the promoter array contains a total of 2,400 promoter PCR products. This represnts a significant increase in genome coverage and allows their use in identifying Egr1 target promoters.

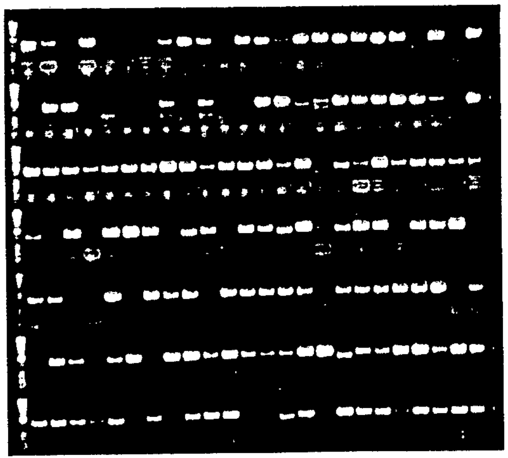

Figure 5. Amplification of gene promoters. 96 well format of gene promoters showing a 90% success rate of amplification. PCR products from a 96 well amplification were electrophoresed on a 1% agarose gel also in 96 well format allowing convenient record keeping. The first lane in each row are molecular weight markers. PCR primers for each promoter have been designed to amplify a product of approximately 1.2 kb.

The promoter arrays are currently being used for hybridization with Egr1 crosslinked and amplified DNA. We are generating multiple sets of independent experiments to perform an analysis of those promoters that show a consistent signal. In addition, I am further expanding the promoter array to at least double the number of promoters represented on the array. I believe that this approach will yield valuable information not only for Egr1 targets, but may also be exploited for the analysis of any other transcription complex. As such, I believe the promoter array will become a highly desirable tool for many investigators.

Appendix

1. Key research accomplishments:

Further characterization of TOE1 as a uv induced phosphoprotein. Phosphorylation enhances the interaction with p53. Coexpression of TOE1 with p53 is able to increase the transactivation potential of a p53 target gene.

Further development of the promoter array as a tool for an array based screen for Egr1 target genes captured by ChIP.

2. Reportable outcomes:

Manuscript (attached)

de Belle, J.X. Wu, S. Sperandio, D. Mercola, and E.D. Adamson.

In vivo cloning and characterization of a new growth suppressor protein TOE1 as a direct target of Egr1 (2003) J. Biol. Chem. 278: 14306-14312.

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In Vivo Cloning and Characterization of a New Growth Suppressor Protein TOE1 as a Direct Target Gene of Egr1*

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Ian de Belle‡§, Jie-Xin Wu‡, Sabina Sperandio¶, Dan Mercola‖, and Eileen D. Adamson‡**

*From the ‡Burnham Institute, La Jolla Cancer Research Center, La Jolla, California 92037, the ¶Buck Institute for Age Research, Novato, California 94945, the ‖Sidney Kimmel Cancer Center, San Diego, California 92121, and the **Cancer Center, University of California at San Diego, La Jolla, California 92093*

Egr1, an immediate early transcription factor, responds to diverse stimuli and affects gene transcription to accomplish its biological effects. One important effect of Egr1 expression is to decrease the growth and tumorigenic potential of several tumor cell types. To identify important Egr1 target genes, we have adapted a methodology involving formaldehyde-induced protein-DNA cross-linking, chromatin immunoprecipitation, and multiplex PCR. Using this approach, we report the cloning of a new Egr1 target gene that is able to account, at least in part, for the growth inhibitory activity of Egr1. We have named this new protein TOE1 for target of *Egr1*.

A common feature associated with the expression of immediate-early genes is their rapid, transient response to a diverse variety of extracellular signals. We have been studying the properties of the early growth response gene, Egr1, which can be transcriptionally induced by a wide spectrum of stimuli including growth factors, cytokines, stresses, depolarizing stimuli, phorbol esters, vascular injury, and irradiation, both ionizing and nonionizing, in a rapid and transient manner with kinetics mirroring those of c-*fos* (1). We have previously presented evidence suggesting a role for Egr1 in suppressing tumor cell growth (2, 3). Specifically, we demonstrated that overexpression of Egr1 in transformed cells suppresses growth in soft agar as well as inhibits their tumor formation in nude mice. Furthermore, it was shown that the DNA-binding domain of Egr1 is necessary for its ability to suppress tumor formation, highlighting the importance of its transactivation of downstream genes in this process (4). Together these results indicate that transformed cells can be induced to revert to normal growth patterns following the re-expression of Egr1. These studies suggest that the loss of Egr1 may result in the loss of cellular homeostasis because of a deficit in Egr1-responsive genes and that this may play a pivotal role in tumorigenesis. Clearly, the identification of a genetic profile of Egr1-responsive genes would constitute a significant step in understanding the different activities associated with Egr1, including its role in cellular growth control. Over the past several years there have been numerous studies identifying various individual Egr1 target genes in diverse cell and tissue types. Reported Egr1 targets include TGF-β1,[1] platelet-derived growth factors A and B, basic fibroblast growth factor, tissue factor, interleukin 2, and CD44 to mention only a few (reviewed in Ref. 5). These studies have focused on the *in vitro* analysis of an individual target gene in a specific cell type under a defined set of experimental conditions. As a step toward a more complete understanding of the biological role for a transcription factor, it would be informative to be able to identify *in vivo* target genes.

Currently, few techniques are available to address this issue. Both differential display and subtractive hybridization analyses are aimed at isolating messages that are up- or down-regulated from pools of RNA isolated from cells or tissues either positive or negative for the gene in question. One clear drawback with both of these techniques is that they select for any RNA message that shows a change in expression pattern. Therefore, when screening for changes in gene expression induced by a transcription factor, these methods do not select purely for direct targets. Recently we and others have described a method for the direct isolation of protein-bound DNA involving *in vivo* chemical cross-linking using formaldehyde followed by immunoprecipitation from chromatin (ChIP). This method was successfully used in applications ranging from examining chromatin structures surrounding the polycomb group proteins during *Drosophila* development (6) and the identification of nuclear matrix attachment sites (7) to the isolation of DNA sequences bound by Egr1 (8). In addition, the same cross-linking method has been used to examine nucleosomal structure, transcription factor occupancy of promoter sites, regions of histone acetylation, and mapping of telomere silencing protein binding, illustrating its broad application utility (9–12). Recently, coupling the ChIP approach with hybridization to genomic or promoter region DNA microarrays has allowed a comprehensive characterization of *in vivo* transcription factor DNA binding patterns (13–16).

In this report we have extended ChIP technology, allowing gene discovery of Egr1 target genes by multiplex PCR. Moreover, we present the cloning of a newly identified gene, called *TOE1*, as an Egr1 target gene. We have characterized TOE1 as a cell growth inhibitor by altering the cell cycle through the induction of p21. Furthermore, we show that the increase in the p21 level is consistent with a mechanism involving TGF-β1.

MATERIALS AND METHODS

Cells, Transfection, Antibodies, and Growth Assays—Both the H4 clone derived from the human fibrosarcoma cell line HT1080 and the

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Ian de Belle and Sabina Sperandio dedicate this manuscript to the memory of Ted and Marilyn Crain.

§ To whom correspondence should be addressed. Tel.: 858-646-3100 (ext. 3650); Fax: 858-646-3198; E-mail: idebelle@burnham.org.

[1] The abbreviations used are: TGF, transforming growth factor; ChIP, chromatin immunoprecipitation; RT, reverse transcriptase.

This paper is available on line at http://www.jbc.org

Egr1 stably transfected H4 subclone E9 have been previously described (4). 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All of the DNA transfections were performed using LipofectAMINE 2000 (Invitrogen), following the manufacturer's instructions. Antibodies against cdc2, phospho-cdc2(Y15), and phospho-p53(S15) were from Cell Signaling Technology. Antibodies against cyclin B1, p21, and p53 were from Santa Cruz Biotechnology. Anti-actinin and the M2 monoclonal anti-FLAG antibody were from Sigma. For cell growth assays 20 × 10^3 control and TOE1 expressing 293 selected and pooled clones were seeded into 96-well plates in triplicate. At the indicated times, cell growth was determined using the CellTiter Cell Proliferation Assay (Promega).

In Vivo Formaldehyde Cross-linking and Chromatin Immunoprecipitation—Cross-linking and chromatin immunoprecipitation were performed as previously described (6, 8). Briefly, the cells were grown in 150-mm plates to 80–90% confluence and then cross-linked by the addition of buffered formaldehyde to a final concentration of 1%. Following exposure to formaldehyde at room temperature for a period of 30 min, the cells were lysed by sonication and chromatin purified by centrifugation through a 5–8 M urea gradient in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). Purified chromatin was dialyzed against 10 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5% glycerol to remove the urea. Samples of 30–60 μg of chromatin were digested with 10 units of *Eco*RI overnight at 37 °C and then precleared by the addition of nonimmune rabbit serum and protein A-Sepharose beads. The precleared samples were immunoprecipitated with affinity purified anti-Egr1 antibodies and protein A-Sepharose beads (17). DNA fragments cross-linked and co-precipitating with Egr1 were purified and ligated to *Eco*RI linkers consisting of 5′-AATTCGAAGCTTGGATCCGAGCAG-3′ and 5′-CTGCTCGGATCCAAGCTTCG-3′. Following ligation, the products were amplified using the 20-mer as primer. Amplification conditions were 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 4 min for 30 cycles. For direct amplification of the ChIP samples, no linker ligation was performed, and direct amplification from the Egr1 immunoprecipitates was done using specific primers for TOE1 (see below), TGF-β, and cyclophilin. The TGF-β primers used were 5′-GGGCTGAAGGGACCCCCCTC-3′ and 5′-TCCTCGGCGACTCCTTCCTC-3′. The cyclophilin primers used were 5′-CTCCTTTGAGCTGTTTGCAG-3′ and 5′-CACCACATGCTTGCCATCC-3′.

Library Multiplex PCR and TOE1 cDNA Cloning—Following amplification of linker-ligated products as described above, the linkers were removed by *Eco*RI digestion, and the products were purified using a PCR product purification kit (Roche Molecular Biochemicals). Multiplex PCR was performed using 100 ng of PCR products as the 5′ primer mix and a T7 oligonucleotide as the 3′ primer, with 100 ng of an excised undifferentiated NT2 cell cDNA library (Stratagene). 30 cycles of hot start PCR were performed using the following parameters: 95 °C for 45 s, 55 °C for 30 s, and 72 °C for 4 min. A 2-kilobase pair band derived from the multiplex PCR was excised from the gel, eluted, cloned into the pCR3.1 TA cloning vector (Invitrogen), and sequenced. Data base homology searches were performed using the BLAST program. To confirm the full-length TOE1 cDNA, we performed 5′ rapid amplification of cDNA ends using the fetal brain Marathon-Ready cDNA kit (Clontech), following the manufacturer's instructions. The TOE1 specific primer used for 5′ rapid amplification of cDNA ends was 5′-GTGAGGGGTACAGCTTTGCC-3′. A FLAG-tagged TOE1 expression vector was generated by PCR using the following primers: 5′-CCGAAGCTTATGGATTACAAGGACGACGACGATAAGGCCGCCGACAGTGAC-3′ incorporating the FLAG epitope tag and 5′-CCGGAATTCTCAGCTACTGCCCCAA-3′. PCR was performed for 30 cycles of 95 °C for 45 s, 62 °C for 30 s, and 72 °C for 2 min. The PCR product was digested with *Hin*dIII/*Eco*RI and cloned into the same sites in pcDNA3. All of the constructs were sequence-confirmed.

Cloning of the TOE1 Proximal Promoter and Luciferase Assays—The proximal region of the TOE1 cDNA sequence was cloned from human genomic DNA using the Advantage-GC genomic PCR kit (Clontech). Primers used for PCR were 5′-GCCGGTACCCGCTCTTACACC-3′ and 5′-CCCGTTAACGACACCGCTCGT-3′. The PCR parameters used were 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 1 min for a total of 30 cycles. This reaction generated a 580-bp product immediately 5′ of the initiation codon. The PCR product was digested with *Kpn*I and *Hpa*I and cloned into the *Kpn*I and *Sma*I sites of pGL3basic (Promega). 293 cells were transfected in 12-well plates with a total of 500 ng of DNA using LipofectAMINE 2000 (Invitrogen). Transfected DNA consisted of 200 ng of expression vector DNA, 200 ng of reporter DNA, and 100 ng of cytomegalovirus-β-galactosidase DNA for normalization. 24 h after transfection, the luciferase assays were performed as described (8).

Mutagenesis—To generate the TOE1 expression construct without the putative nuclear localization signal, QuikChange mutagenesis (Stratagene) was performed. The primers used were 5′-GCGGCAGAGGACGCTTTATTGAACCTA-3′ and 5′-TAGGTTCAATAAAGCGTCCTCTGCCGC-3′. Construction of the correct deletion was confirmed by sequencing.

Gel Shift—The gel shift assay was performed as previously described (8) using the 580-bp radiolabeled TOE1 promoter region described above and recombinant Egr1 protein.

Confocal Microscopy—Control and TOE1 expressing H4 cells were dually stained with rabbit anti-FLAG (Affinity Bioreagents) and mouse anti-nucleolin (Santa Cruz Biotechnology) antibodies. Secondary labeling was performed using fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) and Texas Red-conjugated goat anti-mouse IgG (Jackson Immunoresearch).

Flow Cytometry—The cells were harvested and fixed in 70% methanol and stored at −20 °C until all of the samples were collected. The cells were collected by centrifugation at 2000 × *g* for 3 min, and the cell pellets were suspended in phosphate-buffered saline, digested with RNase A, and stained with propidium iodide.

Northern Blotting—A human Multiple Tissue Northern blot (Clontech) was hybridized with a PCR-generated TOE1-specific ^{32}P-labeled probe using the primers 5′-AAGCGGCGACGGCGACGACG-3′ and 5′-GTGAGGGGTACAGCTTTGCC-3′ following the manufacturer's instructions.

RT-PCR—To detect TOE1 expression following Egr1 transfection, total RNA was harvested from transfected cells using Tri Reagent (Molecular Research Center). Following DNase I treatment, 2 μg of RNA was used for reverse transcription using Moloney murine leukemia virus reverse transcriptase (New England Biolabs). TOE1 expression was then assessed by PCR using the same primers described above for Northern probe preparation, and glyceraldehyde-3-phosphate dehydrogenase expression was determined as a loading control using the primers 5′-AACCATGAGAAGTATGACAAC-3′ and 5′-GTCATACCAGGAAATGAGCT-3′. Expression of the p21 gene was determined using the primers 5′-CTCAAATCGTCCAGCGACCTT-3′ and 5′-ACAGTCTAGGTGGAGAAACGGGA-3′. TGF-β1 expression was assessed using the primers 5′-GCCCTGGACACCAACTATTGCT-3′ and 5′-AGGCTCCAAATGTAGGGGCAGG-3′, and cyclophilin A was amplified using the primers 5′-CTCCTTTGAGCTGTTTGCAG-3′ and 5′-CACCACATGCTTGCCATCC-3′. PCR conditions were 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min for 25 cycles.

Real time PCR reactions were performed using the one-step RT-PCR SYBR green kit from Roche using a Roche Light Cycler instrument. Following the RT reaction for 30 min, the PCR conditions were 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s for 40 cycles. mRNA quantitation was performed by measuring cyclophilin mRNA levels against a standard curve measurement of cyclophilin mRNA from a control sample. The primers used are described above.

In Vitro Kinase Assay—*In vitro* phosphorylation was performed as described (18).

RESULTS

Cloning of TOE1—We have previously characterized a clone of HT1080 cells, called H4, as a cell line that does not express either basal or UV-induced Egr-1. We have also described a series of stable transfected Egr1 clones (19). We used the clone with the maximum expression of Egr1, termed E9, to isolate and identify *in vivo* Egr1 target genes. We performed formaldehyde cross-linking on untreated and UV-stimulated cells followed by chromatin immunoprecipitation as described earlier (8). Because it is generally accepted that Egr1-binding sites usually occur within the proximal promoter region of genes, our immunocaptured Egr1-bound sequences are likely to consist of predominantly promoter regions with extensions into the 5′-untranslated region and even into the coding region. To identify target gene sequences we performed multiplex PCR using our immunocaptured Egr1-bound DNA sequences as 5′ multiplex primers. As template we selected a cDNA library and used a T7 primer that anneals 3′ to all cDNAs permitting full-length cDNA amplification. Using DNA captured from E9 cell Egr1 immunoprecipitates, we found that multiplex PCR-amplified products only in the presence of the multiplex primers, cDNA library, and the 3′ T7 primer (Fig. 1*A*, *lane 2*). When multiplex primers derived from UV-treated E9 cells were used, on occa-

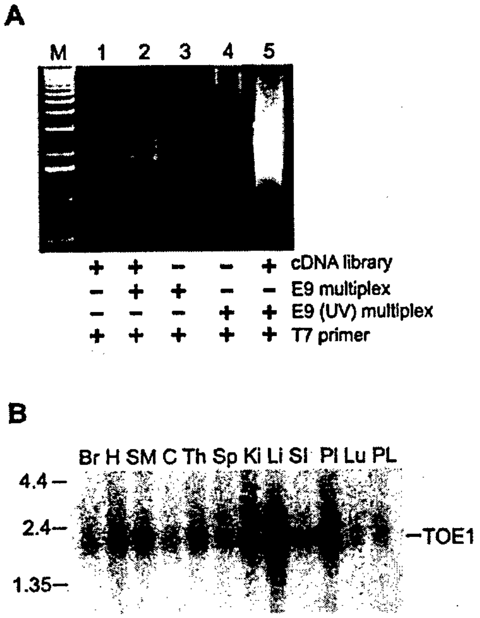

FIG. 1. **Cloning and characterization of the newly identified Egr1 target gene *TOE1*.** *A*, multiplex PCR amplification of Egr1 target genes from a NT2 cDNA library. Linker PCR amplification products of DNA from Egr1 containing immunoprecipitates were used as multiplex primers in a PCR reaction containing a NT2 cell cDNA library template as well as the T7 3′ primer. cDNA amplification products are seen in *lanes 2* and *5*, where all components are present. Both control and UV-treated E9 cells produced PCR products. *Lane M*, 1-kilobase pair DNA markers. *B*, multiple tissue Northern blot hybridized with a *TOE1* probe shows expression of an approximate 2-kb message in adult human tissues. The nucleotide sizes are indicated to the *left*. *Br*, brain; *H*, heart; *Sm*, skeletal muscle; *C*, colon; *Th*, thymus; *Sp*, spleen; *Ki*, kidney; *Li*, liver; *SI*, small intestine; *Pl*, placenta; *Lu*, lung; *PL*, peripheral leukocytes.

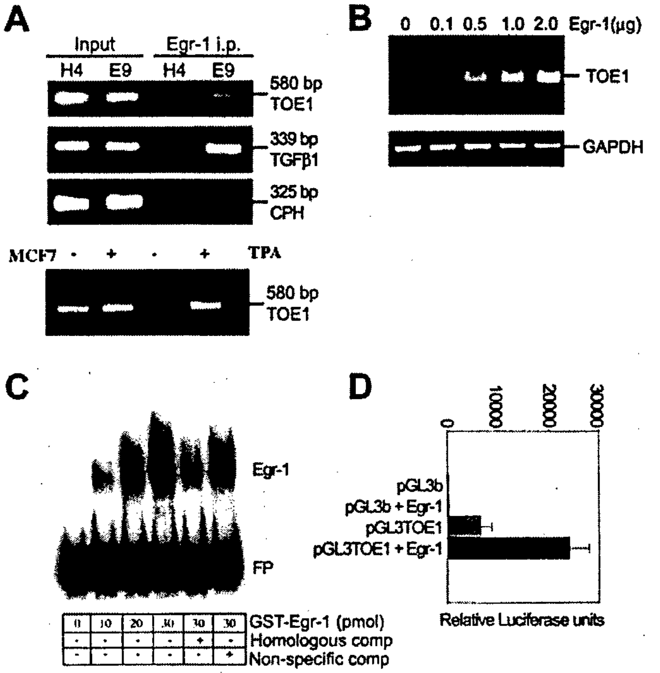

FIG. 2. **Egr1 binds to the 5′ region of *TOE1* and activates its expression.** *A*, PCR amplification of the *TOE1* 5′ region from cross-linked chromatin. Either total cross-linked chromatin (*Input*) or Egr1 immunoprecipitates (*Egr1 i.p.*) were screened directly for the presence of *TOE1* 5′ sequences by PCR using primers designed to amplify a 580-bp fragment 5′ of the initiation codon. The same samples were also used for amplifications using primers for TGF-β1 and cyclophilin A. The same primers were used to analyze Egr1 immunoprecipitates from untreated or 12-*O*-tetradecanoylphorbol-13-acetate-treated MCF7 cells. *B*, Egr1 expression activates *TOE1* expression. RT-PCR amplification of TOE1 from Egr1 transfected H4 cells. Increasing amounts of Egr1 (shown above the lane) were transfected into H4 cells, and total RNA was prepared 24 h later to perform RT-PCR for *TOE1*. Primers within the coding sequence of *TOE1* were designed to amplify a 454-bp product. An equal RNA loading in the RT-PCR reaction was determined using primers amplifying glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *C*, Egr1 binds directly to the TOE1 5′ region. The 580-bp region upstream of the initiation codon of *TOE1* was used as a probe in the gel shift assay. Increasing amounts of recombinant Egr1 showed the binding to this region. Specific binding was determined by adding either unlabeled homologous probe DNA or nonspecific DNA at a 50-fold molar excess. The positions of the free probe (*FP*) and Egr1 shift (*Egr1*) are indicated. *D*, Egr1 transactivates expression from the *TOE1* 5′ region. The same 580-bp 5′ sequence from TOE1 was cloned into the pGL3basic luciferase reporter. Empty reporter vector or the TOE1 reporter in the presence or absence of co-transfected Egr1 expression vector were transfected into 293 cells. 24 h later the cells were harvested and analyzed for luciferase activity. The results have been normalized for transfection efficiency as determined by β-galactosidase measurements. The results are plotted as the average values ± standard deviations. The experiment was repeated three times with similar results.

sion we found some self-amplification from the multiplex primers resulting in a high molecular weight smear (Fig. 1*A*, *lane 4*). However, the addition of cDNA library template produced a much stronger and distinctly different profile of amplified products (Fig. 1*A*, *lane 5*), suggesting that cDNAs were obtained from these primers as well. To directly address the question of whether these amplified cDNAs represented *bona fide Egr1* target genes, we isolated and cloned an individual target gene.

We focused on the distinct DNA band amplified using primers isolated from E9 cells and migrating with an approximate size of 2 kb (Fig. 1*A*, *lane 2*). Cloning and sequencing of this DNA revealed an open reading frame coding for a predicted polypeptide of 510 amino acids and with a predicted molecular mass of ~58 kDa. To confirm that this clone represented a full-length cDNA, we performed 5′ rapid amplification of cDNA ends. Sequencing results confirmed that the captured sequence represented a full-length cDNA clone. A data base homology search of the DNA sequence identified the chromosomal map position on human chromosome 1 (1p34.1–35.3). Comparison of the sequence of this region of chromosome 1 to our cloned cDNA identified an 8 exon gene. BLAST homology searches (20) revealed no extended homology with any known protein. However, a potential single zinc finger was noted as well as a possible nuclear localization signal.

To show that the clone represented an expressed gene, a multiple tissue Northern blot was hybridized and showed intense hybridization to a 2-kb mRNA species in six of the 12 tissues with the highest level of expression in placenta, liver, and kidney (Fig. 1*B*). We cloned the open reading frame of the cDNA, together with a FLAG epitope tag, into a mammalian expression vector and transfected the construct into H4 cells. Western analysis of cells transfected with the FLAG-tagged expression vector and anti-FLAG antibodies showed that the expressed protein migrated on SDS-PAGE with a molecular mass of ~60 kDa, in close agreement with its predicted mass of 58 kDa (data not shown).

TOE1 Is a Target for Egr1 Binding and Transactivation—To confirm the specificity of Egr1 binding to *TOE1 in vivo*, DNA recovered from immunoprecipitates was PCR-amplified to detect the 5′ region of *TOE1*. As shown in Fig. 2*A* we were able to amplify *TOE1* from E9 but not from H4 immunoprecipitates. We did, however, confirm the presence of the *TOE1* gene in the total chromatin fraction, thus ruling out the formal possibility that the *TOE1* gene is deleted in H4 cells. Further, the known Egr1 target gene TGF-β was also amplified from E9 cells (21). The lack of amplification of cyclophilin sequence served as a negative control. This provided evidence that *TOE1* was indeed a target of Egr1 in these cells and that the immunoprecipitated

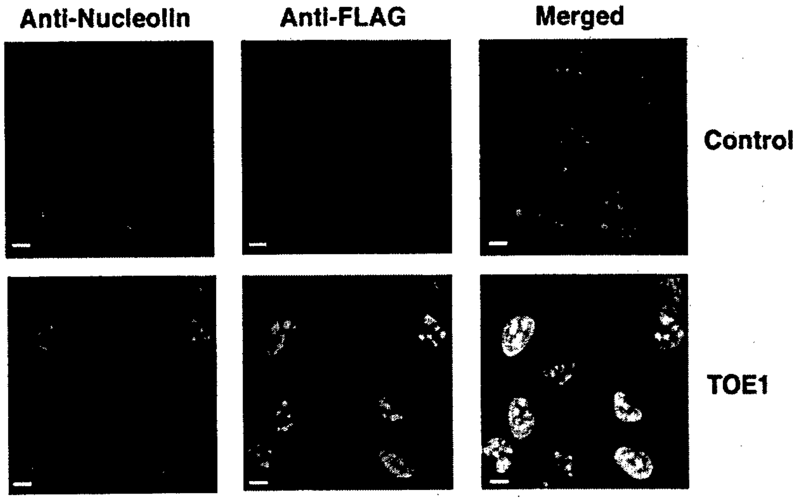

FIG. 3. **TOE1 is a nuclear/nucleolar protein.** Control vector and a FLAG-tagged *TOE1* expression vector were transfected into H4 cells. The cells were immunostained with antibodies to FLAG and to the nucleolar protein nucleolin. Texas Red and fluorescein isothiocyanate-labeled secondary antibodies were used to label nucleolin and FLAG, respectively. Confocal microscopy was performed showing nucleolar co-localization of TOE1 and nucleolin. The *bar* in each *panel* represents 10 microns.

DNA included the 5′ region of the gene. Because E9 cells constitutively overexpress Egr1, we sought to determine whether TOE1 is an Egr1 target in an alternate cell type upon transient Egr1 induction. MCF7 cells were stimulated to express Egr1 by 12-*O*-tetradecanoylphorbol-13-acetate treatment, and then the ChIP assay was performed on untreated or 12-*O*-tetradecanoylphorbol-13-acetate-treated cells. The results shown in Fig. 2*A*, TOE1 was also an Egr1 target gene in these cells. To determine the role of Egr1 in regulating the transcription of *TOE1*, we used RT-PCR following transfection with an increasing amount of an Egr1 expression vector and found a proportional increase in TOE1 expression (Fig. 2*B*).

Direct binding of Egr1 to the *TOE1* promoter region was assessed by a gel shift analysis using as probe a region spanning 580 bp upstream of the translation start. Using recombinant Egr1 we found specific binding to the probe (Fig. 2*C*). When oligonucleotides representing the consensus Egr1-binding site were used as competitor, effective competition was also observed (data not shown). As a test of the functional properties of the complex we inserted the same 580-bp 5′ region upstream of a luciferase reporter. We observed that this region responds to Egr1 expression by activating transcription (Fig. 2*D*). Together, these results are consistent with *in vivo* binding of Egr1 to and transactivation of the *TOE1* gene.

Subcellular Localization of TOE1—To determine the intracellular localization of TOE1, a FLAG-tagged expression construct was transfected into H4 cells. As shown in Fig. 3, following immunostaining for the FLAG epitope, the subcellular localization of TOE1 was distinctly nuclear. Transfection and staining of H4 and 293 cells (not shown) showed patterns of concentrated localization within the nucleus. These sites of concentration appeared to correspond to nucleoli. Dual staining using anti-FLAG and anti-nucleolin antibodies followed by confocal microscopy (Fig. 3) showed that most of the expressed TOE1 co-localized with nucleolin, indicating a predominant nucleolar location for TOE1. In addition to its nucleolar localization we observed intense staining for TOE1 as multiple nuclear speckles. As noted above, data base homology searches identified a putative nuclear localization sequence consisting of KRRRRRRREKRKR located at positions 335–347 in the 510-amino acid protein. Deleting the putative nuclear localization basic stretch of amino acids resulted in the cytoplasmic localization of TOE1 (Fig. 4), suggesting that this sequence is responsible for TOE1 nuclear targeting.

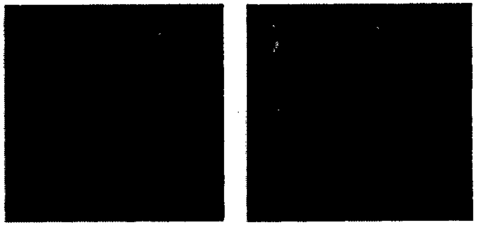

FIG. 4. **Identification of TOE1 nuclear localization sequence.** H4 cells were transfected with either a FLAG-tagged wild type TOE1 expression vector (*left panel*) or a FLAG-tagged TOE1 expression vector containing a deletion in the putative nuclear localization sequence (*right panel*). Following fixation, the cells were subjected to immunostaining using anti-FLAG (*red*). For the cells expressing the TOE1 nuclear localization sequence deletion, the nuclei were counterstained with 4′,6-diamidino-2-phenyl.

TOE1 Expression Affects the Growth of 293 and H4 Cells—To test whether TOE1 might be involved in mediating the growth effects of Egr1, we measured the growth rate of cells stably transfected with a TOE1 expression vector. Fig. 5*A* shows that the growth rate of TOE1-expressing cells was severely reduced in comparison with empty vector control cells. The doubling time for control cells was ~24 h, whereas a pool of TOE1 expressing clones required 40 h to double in number. Transfection of the same vector expressing the calcium binding protein calbindin had no effect on cell growth (data not shown), suggesting that inhibition by TOE1 was not a nonspecific effect of protein over expression. Similar results were obtained in H4 cells (data not shown).

Cell growth inhibition in TOE1-expressing cells was also examined by performing colony forming assays. Control cells formed numerous rapidly growing colonies, whereas TOE1-expressing cells were only able to form 30% as many colonies (data not shown). To determine whether the decrease in cell growth of TOE1-expressing cells represented a generalized slowing of growth or a cell cycle stage-specific slowing, we performed flow cytometry on log phase cells. We found a significant increase in the fraction of cells present in the G_2/M phases of the cell cycle in TOE1-expressing cells (27%), compared with the control cells, with 13% of the cells in this fraction (Fig. 5*B*). We found no difference between the mitotic index of control and TOE1-expressing cells, suggesting that TOE1 was pausing the cells in the G_2 phase (data not shown). In addition, it should be noted that we found TOE1 expression to be highly influenced by the growth state of the cells. Specifically, we have found TOE1 expression to be regulated by cell culture density, possibly indicating a form of activation caused by contact inhibition.[2] The expression of TOE1 in dense cell cultures occurred even in cells that cannot express Egr1, indicating that although Egr1 can activate expression of TOE1, the

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[2] I. de Belle and J.-X. Wu, unpublished observation.

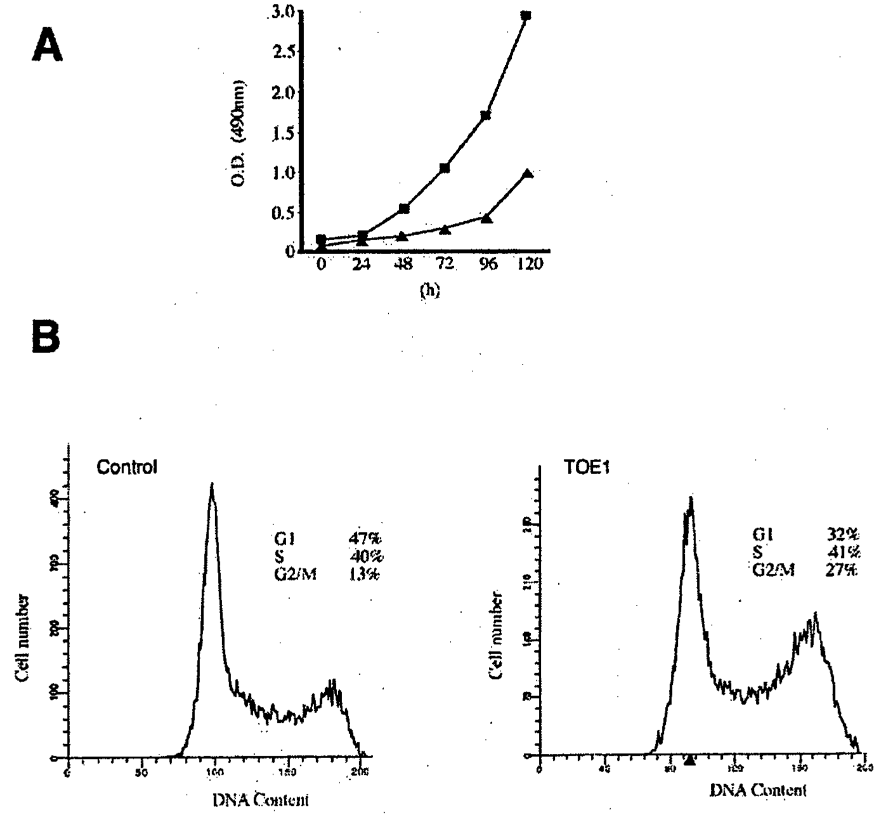

FIG. 5. **TOE1 expression affects cell growth and the cell cycle.** *A*, TOE1 decreases the growth rate of 293 cells. Pooled clones of empty vector or *TOE1*-expressing cells were used to determine their growth rate over a period of 5 days. *Solid squares*, control transfected cells; *solid triangles*, *TOE1*-expressing cells. The results are the averages of triplicate readings, and the experiment was repeated three times with similar results. *B*, *TOE1* expression affects the cell cycle. The cell cycle distribution of log phase growing control and TOE1 expressing clones of H4 cells was determined by flow cytometry. The calculated percentages of the cell cycle phases are indicated.

gene must be subject to additional forms of regulation.

TOE1 Causes an Increase in p21 Expression in H4 Cells—To investigate the mechanism of TOE1 induced G_2 phase delay, we performed Western blotting on several G_2 cell cycle markers. Fig. 6*A* shows that there was no significant change in cyclin B1, cdc2, or phospho-cdc2 levels between control, TOE1, and mutant TOE1-expressing cells (with the nuclear localization deleted). This suggested that the activation potential of the G_2-specific CDK complex was unaffected by the expression of TOE1. We therefore examined the possibility that the activity of the complex might be modulated by its known inhibitor p21. The level of p21 was dramatically up-regulated in TOE1-expressing cells but not in either control or TOE1 mutant cells. Because p53 is a known transactivator of the p21 gene, we examined the level and activation of p53 in our cells. We were unable to find a significant induction or activation of p53, at least insofar as serine 15 phosphorylation is concerned. Further exploration of the induction of p21 using RT-PCR showed that TOE1-expressing cells up-regulated p21 at the mRNA level (Fig. 6*B*). This activation was not seen in cells expressing non-nuclear mutant TOE1. To demonstrate that the increase in p21 was functionally associated with an effect on cdc2 activity, we immunoprecipitated cyclin B1 and measured the associated kinase activity *in vitro* with histone H1 as substrate. Fig. 6*C* shows a significant decrease in kinase activity only in TOE1-expressing cells, correlating with increased p21 expression in those cells.

Increased TGF-β1 in TOE1-expressing cells—Because Egr1 expression is known to affect TGF-β1 levels (21), we sought to determine whether the increase in p21 levels might be mediated by TGF-β1. Using real time quantitative PCR, we examined the TGF-β1 levels in cells transfected with a TOE1 expression vector. As shown in Fig. 7, using both MCF7 and H4 cells lines, we noted an increase in the level of TGF-β1 mRNA in TOE1 transfected cells compared with control transfected cells.

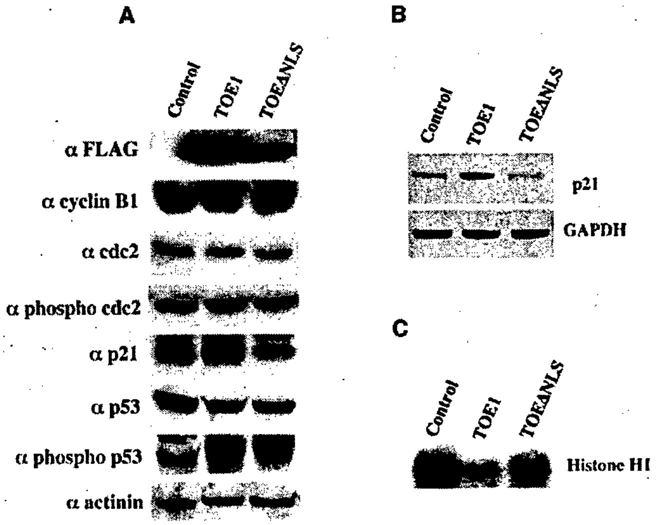

FIG. 6. **TOE1 affects growth inhibition through increased p21 expression.** *A*, control, TOE1, and TOE1ΔNLS cells were probed by Western blotting with the indicated antibodies. *B*, RNA was extracted from cells, and RT-PCR was performed for the expression of p21 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *C*, *in vitro* phosphorylation assay. Cyclin B1 immunoprecipitates were incubated with histone H1 and radiolabeled ATP. The products were visualized by SDS-PAGE and autoradiography.

DISCUSSION

With these studies we report, for the first time, the application of chromatin immunoprecipitation to cDNA cloning using a form of multiplex PCR. We have demonstrated that this technique was successful not only in cloning transcription factor target genes but also in the identification of a new target for Egr1. Together our results indicated that the multiplex amplification produced a genuine cDNA and that the cloned DNA represented an expressed gene. This newly cloned gene encodes

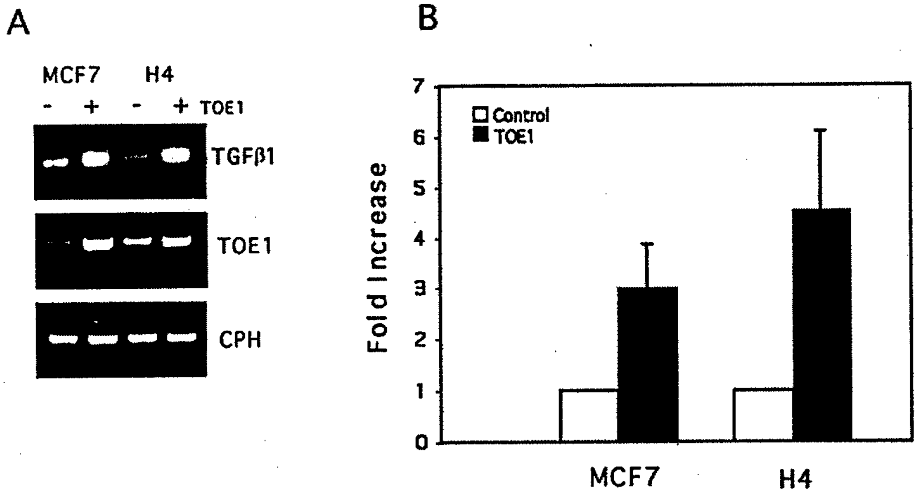

FIG. 7. **TOE1 expression affects the levels of TGF-β1 mRNA.** *A*, RT-PCR measurement of TGF-β1 and TOE1 from MCF7 and H4 cells transfected with an empty vector control (−) or a TOE1 expression vector (+). 24 h after transfection, RNA was harvested from the cells, and RT-PCR was performed using the protocol described under "Materials and Methods" with 25 cycles of amplification. Cyclophilin A amplification was used to demonstrate the equal RNA amounts included in each reaction. *B*, real time quantitative PCR was performed on MCF7 and H4 cells transfected with either control empty vector or a TOE1 expression vector. 24 h following transfection, RNA was collected and subjected to real time PCR for TGF-β1 mRNA quantitation. The *open bars* represent the relative quantity of TGF-β1 level in control cells, and the *closed bars* represent that for TOE1 transfected cells. mRNA samples were normalized to cyclophilin A levels. The results shown are the averages of four independent experiments showing standard deviations.

a 510-amino acid protein that we have shown to be an authentic Egr1 target gene. To confirm that the gene codes for an endogenously expressed protein, we have recently raised a polyclonal antibody using a synthetic peptide epitope derived from the predicted amino acid sequence. Preliminary testing has shown reactivity against both recombinant and an endogenous protein of identical molecular mass, suggesting that the cDNA is expressed at both the mRNA and protein level.

During the course of these studies an unpublished and unnamed cDNA generated through a library sequencing effort was deposited in the GenBank™ data base that was identical to our cloned cDNA (nucleotide accession number AK024011). Based on the sum of our observations, we have called this cDNA the HUGO approved name and symbol *TOE1* for target of *Egr1*. Expression of TOE1 was detected in all of the adult human tissues examined but at varying levels, indicating that the regulation of this gene may vary depending on cell or tissue type.

Examination of the sequence of TOE1 did not reveal conserved domain structures apart from a single potential zinc finger and a possible nuclear localization signal. Immunostaining confirmed that TOE1 was found localized to the nucleoplasm and nucleolus. Despite the absence of a recognized DNA-binding domain, we have examined the possibility that TOE1 might participate in transcriptional regulation. However, TOE1 cloned as a GAL4 fusion failed to activate a GAL4-binding site reporter, suggesting that TOE1 alone is not sufficient for transcriptional regulation. The possibility remains that TOE1 can participate in transcriptional regulation through protein interactions and indirect DNA association not recapitulated in the GAL4 fusion experiments. Although no extended homology to any known gene was noted by BLAST searches, a limited region of homology to poly(A)-specific deadenylation nuclease was revealed. We are currently investigating the possibility that TOE1 may function as a nuclease.

To better understand the biological role of TOE1, we examined the effects of its expression and noted a dramatic decrease in both the growth rate and colony growth of H4 cells. We found that this was not the result of a general decrease in growth rate but rather was due to a G_2 cell cycle phase delay. Furthermore, the G_2-specific cell cycle delay correlated with an increase in the expression of the cyclin-dependent kinase inhibitor p21. Deletion of the nuclear localization signal abrogated this effect, suggesting not only that TOE1 could induce cell cycle-specific G_2 pausing but also that its nuclear/nucleolar localization was critical for this function. The localization of TOE1 in the nucleolus may provide further evidence for a role in cell cycle regulation because it has been found that many important cell cycle proteins can be found in the nucleolus as a means of sequestration, thereby limiting their function until the appropriate time (22–24).

Because p21 is also able to inhibit cyclin-dependent kinase activities controlling passage through the G_1 restriction point, it would be predicted that the TOE1-directed increase in p21 levels would also display a G_1 phase pausing. Although we did not see this in log phase growing cells, when cells were synchronized in the M phase and then released to pass through G_1, we noted a marked delay in the TOE1-expressing cells (data not shown). This suggested that the increase in p21 levels was also active at the G_1 check point, but this was only seen if cells had been synchronized outside of the G_2 phase. Although p21 is well known for its activity in G_1 phase pausing, its role in G_2 is being increasingly recognized (25, 26). These results suggest that the mechanism by which TOE1 affects cell growth is through transcriptional up-regulation of the p21 gene. We have not, however, formally ruled out the possibility that the increase in p21 levels might be due to an increase in transcript stability rather than increased expression. Also, we have not completely ruled out a contributing role for p53 in the up-regulation of p21 but have demonstrated that p53 levels and serine 15 phosphorylation were not altered. Further, we have provided evidence that TOE1-dependent TGF-β1 activation may participate in the increase in p21. However, it also remains possible that TOE1 and p53 cooperate in the transactivation of p21 either directly or indirectly. We have preliminary evidence that TOE1 and p53 are able to interact physically, but the significance and specificity of this interaction remain to be analyzed.[3] Although the precise mechanism of action remains to be studied, our results have shown that expression of TOE1 leads to growth inhibition as well as a decrease in colony forming ability, likely involving the activation of p21. Given that these same features are seen following expression of Egr1, we expect that the downstream target TOE1 plays an important role in executing this physiological function of Egr1 in its proposed role as a tumor suppressor.

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[3] I. de Belle, unpublished observation.

Finally, It is intriguing to note that the chromosomal location of *TOE1* maps to 1p34.1–35.3. Deletion of the distal portion of 1p accounts for a significant proportion of chromosome 1 aberrations and has been observed in brain, breast, ovarian, colorectal, and other tumor types (27–29). Combined data suggest that chromosome 1p likely harbors one and possibly multiple tumor suppressor genes, and given the growth inhibitory effect of TOE1, we are currently investigating the possibility that TOE1 may also function in this capacity.

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